Epitope Mapping of Anti-recA Protein IgGs by Region Specified Polymerase Chain Reaction Mutagenesis*

Masayuki Ikeda, Kaoru Hamano, and Takehiko Shibata‡

From the Laboratory of Microbiology, RIKEN (The Institute of Physical and Chemical Research), Wako-shi, Saitama 351-01, Japan

Monoclonal IgGs were shown to be useful for the specific inhibition of a set of activities of the recA protein, a key protein in homologous genetic recombination. The mapping of the epitopes for these IgGs and site-directed mutagenesis based on the mapping will facilitate location of the functionally active sites on the tertiary structure of the protein, which is being solved by means of physicochemical techniques. We developed a novel technique for region-specified mutagenesis and applied the technique to epitope mapping. Using the polymerase chain reaction in the presence of deoxyinosine triphosphate, we introduced random base substitutions specifically into a region of the recA gene defined by a pair of primers. RecA mutants exhibiting altered antigenicity were selected, in plaque-immunoblotting experiments, from libraries of mutagenized recA genes constructed on the λgt11 expression vector. Mutant recA genes were obtained at the frequency of about 10^{-2} among the plaques expressing fused recA genes and then each one was expressed as a whole protein, which was characterized by enzyme-linked immunosorbent assay. Analyzing the DNA sequences of the mutant recA genes, we located at the amino acid sequence level the epitopes for two anti-recA IgGs which could not be located in previous studies. One of the antibodies was shown to prevent self-assembly of the recA protein and the other was suggested to inhibit the binding of double-stranded DNA. Thus, the active sites involved in these functions would be located in the space around or near the relevant epitope.

The recA protein, and its prokaryotic and virus (T4 phage) analogues promote "homologous pairing" and "strand exchange" between homologous double-stranded and single-stranded DNAs through ATP (or dATP-)-dependent reactions *in vitro*, and were shown to play an essential role in homologous genetic recombination *in vivo*. Homologous pairing is the formation of an intermolecular duplex ("heteroduplex") between a couple of homologous single-stranded and double-stranded DNAs, and strand exchange is the processing of the heteroduplex, such as its elongation. Each of these reactions consist of a number of substeps and the recA protein or its analogues appear to have various active sites that

promote each of these substeps, such as an ATP-binding site, ATPase catalytic center, binding site for single-stranded DNA, binding site for double-stranded DNA, and sites for self-polymerization. The localization of these active sites on the tertiary structure of the recA protein is essential for understanding the mechanisms of the underlying biochemical functions of the protein. However, only sites related to ATPase have been partly located at the amino acid sequence level. The mapping of mutation sites as well as x-ray crystallographic analysis of the protein are the main means to this end. A series of our studies involving the use of anti-recA protein monoclonal IgGs is also an approach to the same goal (see Shibata *et al.*, 1991, for review).

We have isolated clones of mouse hybridomas which produce anti-recA protein IgGs (Makino *et al.*, 1985). Two (ARM193 and ARM191) of these anti-recA protein IgGs each inhibit a set of activities of the recA protein without affecting the others; *i.e.* ARM193 severely inhibits the unwinding of the double helix and strand exchange, but allows homologous pairing and single-stranded DNA-dependent ATP hydrolysis (Ikawa *et al.*, 1989; Makino *et al.*, 1985, 1987). On the other hand, ARM191 inhibits the homologous pairing and unwinding of the double helix, but only affects the single-stranded DNA-dependent ATP hydrolysis a little (Makino *et al.*, 1985). ARM193 was suggested to affect the site for the interaction between recA polypeptides (Ikawa *et al.*, 1989) and ARM191 to affect the site on the recA polypeptide for the binding to double-stranded DNA (Makino *et al.*, 1985). We preliminarily located the epitopes for both ARM193 and ARM191 in a C-terminal 88 amino acid region (Phe[260]-Glu[347]) of the recA polypeptide by examining the cross-reaction of proteolytic fragments. However, we failed to map them more precisely, since none of the subfragments of the 88-amino acid region exhibited significant cross-reaction with either of the IgGs (Ikeda *et al.*, 1990). Therefore, it was necessary to introduce another technique to overcome the problem. Here, we describe a novel technique for region-specified mutagenesis and, as an application of this technique, the mapping of the epitopes of ARM193 and ARM191 at the amino acid sequence level in distinct but slightly overlapped subregions in the C-terminal 88-amino acid region.

MATERIALS AND METHODS

recA Protein—The purified recA protein was fraction V prepared as described (Shibata *et al.* 1981).

Oligonucleotides—Oligonucleotides were synthesized with a DNA synthesizer (Du Pont-New England Nuclear CODER300) and purified with NENSORB PREP (Du Pont-New England Nuclear).

Techniques for Cloning of DNAs—Treatment with restriction endonucleases and DNA ligase, and the isolation and cloning of the DNA fragments onto vectors were carried out as described (Berger and Kimmel, 1987; Maniatis *et al.*, 1982).

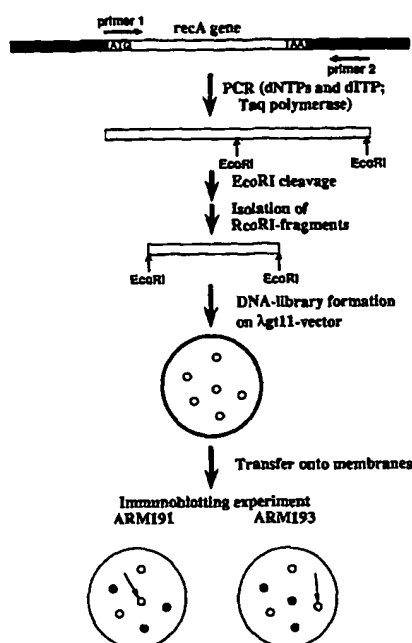*Antibodies and Immunochemical Techniques*—The anti-recA pro-

FIG. 1. Region-specified PCR mutagenesis. DNA encoding the *E. coli* *recA* gene flanked by primers 1 and 2 was amplified by PCR in the presence of dITP with the use of *Taq* DNA polymerase. *Eco*RI fragments of the amplified DNA which encoded the C-terminal region of the recA protein were cloned on a λgt11 expression vector to construct DNA libraries of the mutagenized *recA* genes. With appropriate orientation of a fragment relative to the vector, the C-terminal region of the *recA* gene was connected to the *lacZ* gene in-frame. Proteins expressed in the plaques obtained from the libraries were transferred to a pair of membranes and then the cross-reaction with either anti-recA protein IgG ARM191 or ARM193 was tested. The plaques showing cross-reaction with only one of the IgGs (indicated by *arrows*) were picked up and subjected to further cross-reaction tests. The *closed circles* in the *big circles* at the bottom of the figure denote plaques which showed cross-reaction with the indicated IgG.
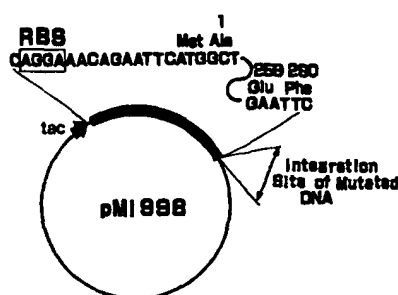

FIG. 2. Structure of pMI996 for expression of mutated *recA* genes. DNA encoding the N-terminal region of the recA polypeptide was put under the control of the *Tac* promoter on a multicopy plasmid (pKK223-3). *Eco*RI fragments of the mutagenized *recA* gene were inserted at the *Eco*RI site at the codons for Glu^{259}-Phe^{260}.

tein monoclonal IgGs, ARM191, ARM193, and ARM414, were described previously. We used affinity purified preparations of these IgGs. An anti-recA protein monoclonal IgG, MAb156, was isolated by Karu and Allen (Karu and Allen, 1982), and a purified preparation of this IgG was provided by Dr. Alexander Karu (University of California, Berkeley) and Dr. A. John Clark (University of California, Berkeley).

The enzyme-linked immunosorbent assay (ELISA)[1] was carried out as described previously. Unless otherwise stated, samples of cell-free extracts were diluted in PBS (50 mM potassium phosphate buffer

___

[1] The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.
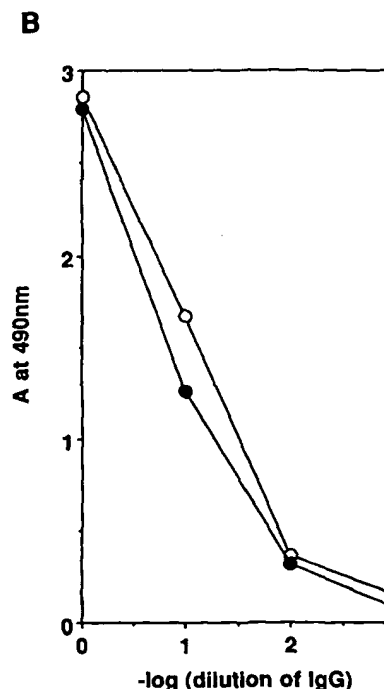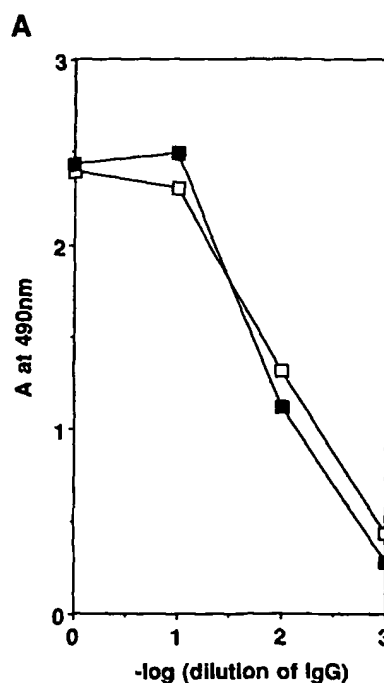
FIG. 3. Absence of competition in cross-reaction between anti-recA protein IgGs, ARM191 and ARM193. The wells of a microtiter plate were coated with the purified recA protein. Then, a solution of a tested anti-recA protein (nondilution: 1.5 µg/ml) containing the other IgG was put into the wells and allowed to cross-react with the recA protein. ARM191 belongs to IgG_1 and ARM193 to IgG_{2b}. Thus, the amounts of ARM191 and ARM193 bound to the recA protein were specifically measured by ELISA with the use of an appropriate subclass-specific antibody. *A*: □, the binding of ARM191 in the absence of ARM193; ■, the binding of ARM191 in the presence of 5 µg of ARM193/ml. *B*: ○, the binding of ARM193 in the absence of ARM191; ●, the binding of ARM193 in the presence of 5 µg of ARM191/ml.

(pH 7.2) containing 150 mM NaCl) and put into the sample wells of a microtiter plate with 96 wells. On the other hand, the concentration of the anti-recA protein IgG was first adjusted to 30 µg of protein/ml ("no dilution") in PBS Tween (PBS containing 0.05% Tween 20) and then a series of dilutions was prepared in PBS/Tween.

FIG. 4. **Testing of cross-reaction of proteins in plaques obtained from libraries of mutagenized DNA.** The results of two experiments are shown as examples (*A* and *B*). *A1* and *B1* show cross-reaction with ARM191, and *A2* and *B2* that with ARM193. The procedure was described in detail in the legend to Fig. 1. *Arrowheads* indicate mutants which showed altered cross-reaction; mutant D (the top ones in *A1* and *A2*) shows no cross-reaction with ARM191, and mutant 47 (*B1* and *B2*) none with ARM193.

TABLE I

Isolation of mutants by region-specified PCR mutagenesis

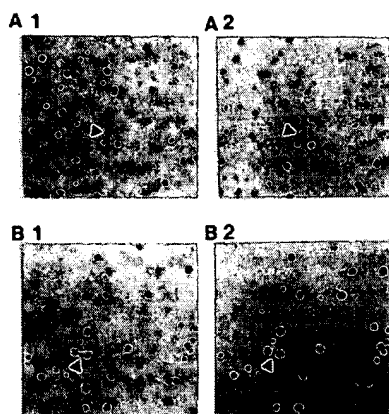| dITP	Total plaques expressing recA genes	Number of plaques picked up on the first selection	Total number of mutants identified	Number of species of mutants
µM				
0	1800	5	1	1
0.2	1100	21	4	2
20	2200	5	3	3
200	2000	21	11	10

Testing of the Competition between ARM191 and ARM193—The IgGs, ARM191 and ARM193, belong to subclasses 1 and 2b, respectively (Ikeda *et al.*, 1990), and thus each could be assayed by use of anti-mouse IgG_1 and anti-mouse IgG_{2b} antibodies, respectively. A solution of the purified recA protein (0.2 µg/ml) was put into the wells of a microtiter plate to coat the walls of the wells. Each solution of an indicated amount (no dilution: 1.5 µg/ml) of a tested anti-recA protein IgG (50 µl) contained 0 or 5 µg of the other anti-recA protein IgG/ml. The amounts of the tested anti-recA protein IgG that bound to the wells were measured by ELISA with anti-mouse IgG_1 or anti-mouse IgG_{2b} antibodies (Zymed Laboratories, Inc.).

Region-specified PCR Mutagenesis—An outline of the procedure is given in Fig. 1. pBEU14 DNA, which contains the *Escherichia coli* recA gene (Uhlin and Clark, 1981), was linearized with *Bam*HI and used as the template for PCR (polymerase chain reaction). A DNA region encoding the recA gene flanked by primer 1 (5'-ATGGCT-ATCGACGAAAACAA-3') and primer 2 (5'-GAATTCTGTCATG-GCATATCCTT-3') was amplified by 25 cycles of PCR (see Fig. 1). Unless otherwise stated, the reaction mixture for PCR comprised 1 µM each of primers 1 and 2, about 3 pM the template DNA, 200 µM each of dATP, dTTP, dGTP, and dCTP, 200 µM deoxyinosine 5'-triphosphate (dITP), 0.025 units of *Taq* DNA polymerase/µl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, and 10 mM Tris-HCl buffer (pH 8.3). Each cycle of PCR consisted of: (i) incubation at 37 °C for 2 min, for loading of the primers onto the template DNA, (ii) incubation at 72 °C for 3 min, for polymerization, and (iii) incubation at 94 °C for 1 min, for denaturation. The amplified DNA was treated with *Eco*RI and the *Eco*RI fragment encoding the C-terminal 93-amino acid region was isolated by gel electrophoresis, followed by trapping on a DEAE membrane (NA45; Schleicher & Schuell). Then, DNA libraries containing the mutagenized *Eco*RI fragments were constructed on the λgt11 expression vector by ligating them at an *Eco*RI site of the vector. With appropriate orientation of a fragment relative to the vector, the C-terminal region of the recA gene was connected to the *lacZ* gene in-frame. Plaques of the phages in the libraries were obtained with the *E. coli* Y1090 strain as a host. Proteins in the plaques were transferred to a pair of membranes and then the cross-reaction with either ARM191 or ARM193 anti-recA protein IgG was

examined by means of immunoblotting experiments, as described previously (Ikeda *et al.*, 1990; Morishima *et al.*, 1990). The plaques showing cross-reaction with only one of the IgGs (indicated by *arrows* in Fig. 1; examples are shown in Fig. 4) were picked up, and the phages were obtained after repeated single plaque isolation and testing by means of immunoblotting experiments.

DNA Sequence Analysis—The tested DNAs were cloned on pUC119 and then their sequences were analyzed by the dideoxyribonucleotide chain termination method (Sanger *et al.*, 1977); the labeling reaction was carried out by use of the double-stranded template according to a manual for Sequenase (United States Biochemical Co., Cleveland, OH), and the products were analyzed with the use of an automated DNA sequence analyzer (Du Pont GENESIS2000). We analyzed both strands in most of the cases.

Expression of Mutant recA Genes and Preparation of Cell-free Extracts—*Eco*RI fragments which carried mutation(s) in the C-terminal 93-amino acid region were ligated to the *Eco*RI site of the DNA region encoding the N-terminal 260 amino acids, which was under the control of the Tac promoter on a multicopy plasmid, pMI996 (Fig. 2), a derivative of pKK223-3 (Brosius and Holy, 1984). An *E. coli* strain, MV1184 (deletion of the *srl-recA* locus) (Vieira and Messing, 1987), was transformed with the DNA. The transformants were grown at 37 °C to the mid-logarithmic growth phase in 3-ml cultures and then expression of the mutated recA genes was induced by isopropyl-β-D-thiogalactoside treatment (0.2 mg/ml) for 2 h. After the treatment, the cells were collected by centrifugation and suspended in 200 µl of a lysis buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 M NaCl, and 5% Triton X-100), and then treated with lysozyme (0.6 mg/ml) at 0 °C for 15 min, followed by the addition of KCl (at 0.24 M). The lysate was centrifuged at about 15,000 × g for 15 min and the supernatant of the lysate was saved. The supernatant was diluted about 20-fold with PBS and then subjected to ELISA as described above. In the case of mutants 2 and 38, the precipitates of the lysates were resuspended in 5 M urea containing 100 mM Tris-HCl (pH 7.9), followed by centrifugation, and the mutant proteins were extracted from the precipitates with 100 mM Tris-HCl buffer (pH 7.9) containing 8 M urea and 0.1 M NaCl at 25 °C for 1 h.

RESULTS

Testing of the Competition between ARM191 and ARM193—The preliminary mapping of the epitopes of ARM191 and ARM193 indicated that both epitopes were located between Phe^{260} and Glu^{347} (Ikeda *et al.*, 1990). First, we examined whether or not these anti-recA protein-IgGs showed competition in the cross-reaction with the recA protein. Since ARM191 and ARM193 belong to different IgG subclasses, each can be discriminated through the use of subclass-specific antibodies on ELISA. Fig. 3 indicates that the presence of one of these IgGs did not affect the binding of the other IgG to the recA protein. We conclude that the epitope for ARM191 and ARM193 are different. Thus, we tried to locate the epitopes of these anti-recA protein IgGs more precisely.

Development of Region-specified PCR Mutagenesis and Isolation of Mutants—Since we were not able to locate the epitopes of ARM191 and ARM193 by examinations of the cross-reaction of subfragments of the recA polypeptide, we developed a novel technique for region-specified mutagenesis which could be applied for mapping of the epitopes. The whole process for the isolation of mutants causing altered cross-reaction consists of three stages (Fig. 1); the introduction of region-specified random base substitutions by means of PCR, construction of libraries of the mutagenized DNA with the use of the λgt11 expression vector, and *in situ* testing for cross-reaction of the mutated polypeptides expressed in the plaques obtained from the libraries. In PCR (Saiki *et al.*, 1985), *Taq* DNA polymerase causes the misincorporation of nucleotides (Eckert and Kunkel, 1990). We added deoxyinosine 5'-triphosphate (dITP), at 200 µM, to the reaction mixture for PCR to enhance the misincorporation (Martin and Castro, 1985), and specifically amplified a DNA region defined by a pair of oligonucleotides (primers 1 and 2). Primer 1 includes the initiation codon of the recA polypeptide and

TABLE II

Mutations affecting cross-reaction with anti-recA protein IgG ARM191 or ARM193

Mutation site on recA polypeptide	Name of mutant	Change in DNA sequence	Amino acid replaced	Cross-reactivity on immuno-blotting[a]		Half-maximum values on ELISA			
				ARM191	ARM193	ARM191	ARM193	ARM414	MAb156
						μg/ml			
Wild type				+	+				
Experiment 1						0.017	0.27	0.12	0.019
Experiment 2						0.085	0.21	0.19	0.017
Experiment 3						0.021	0.75	0.12	0.012
Average of "plus" recA polypeptide						0.023	0.28	0.14	0.015
(S.D.)						(0.024)	(0.18)	(0.11)	(0.004)
283	38	CTG → CCG[b]	Leu → Pro	−	+	0.13	0.17	0.075	>6.0
291	D	TAC → TGC	Tyr → Cys	−	+	>30	0.27	0.24	>30
296	43	GAG → GGG	Glu → Gly	−	+	>30	0.53	0.19	>30
305	4	GCG → GGG	Ala → Gly	−	+	0.75	0.30	0.21	1.7
308	32	TGG → TGC	Trp → Cys	−	+	>30	0.13	0.21	>30
309	2, 5, 12[c]	CTG → CCG	Leu → Pro	−	+	>7.5	0.15	0.053	>30
312	37, 39[c]	AAC → AAA	Asn → Lys	−	+	>9.5	0.19	0.11	>9.5
315	30.1[d]	ACC → ATC	Thr → Ile	+	−	0.013	>10	0.052	0.015
332	31, 33.1[c,d]	AAC → GAC	Asn → Asp	+	−	0.012	>10	0.06	0.013
333	23, 34	TCA → CCA	Ser → Pro	+	−	0.015	>10	0.04	0.019
337	47	TTC → TCC	Phe → Ser	+	−	NT[e]	NT	NT	NT
338	40	TCT → TTT	Ser → Phe	+	−	0.0095	>30	0.075	0.015
294	36.1	AAA → AGA	Lys → Arg	+	−	0.013	>10	0.075	0.0075
330	36.2	AAC → GAC	Asn → Asp						
319	41.1[d]	ATC → ACC	Ile → Thr	−	+	>30	0.085	0.48	>30
323	41.2	GTA → GCA	Val → Ala						
267	30.2, 33.2, 36.3[c]	GGT → GGC	No replacement						
321	30.3	AAG → AAA	No replacement						

[a] Proteins showing the plus phenotype with respect to cross-reaction with the relevant IgG.

[b] Shadow letters indicate bases substituted.

[c] All mutants having the same substitution were isolated from the same PCR preparation, except mutants 23 and 34.

[d] Mutants 30, 33, 36, and 41 have more than two base substitutions, each of which is indicated, e.g. 30.1 and 30.2.

[e] NT, not tested.

primer 2 includes a complementary sequence about 80 bases downstream of its stop codon (Fig. 1). Primer 2 was designed so as to have an EcoRI cutting site. At more than 200 μM, dITP severely inhibited the amplification of DNA by PCR (data not shown). After 25 cycles of PCR, the amplified DNA was treated with EcoRI and the EcoRI fragment encoding the region of the recA polypeptide extending from Phe[260] to the C-terminal Phe was isolated. The fragment was inserted into the EcoRI site of the λgt11 expression vector (Young and Davis, 1983). The proteins in the plaques were examined as to their *in situ* cross-reaction with anti-recA protein IgGs ARM191 and ARM193. As shown in Fig. 4, most of the plaques expressing the fused recA polypeptide showed cross-reaction to both IgGs. We could pick up about 1% of these plaques as candidates of mutants which showed cross-reaction to only one of the IgGs (indicated by *arrowheads* in Fig. 4; Table I).

From the selected plaques, phages were recovered and the inserted EcoRI fragments were reisolated. The DNA fragments were recloned on the pUC119 vector (Vieira and Messing, 1987) and their DNA sequences were analyzed by the dideoxyribonucleotide chain termination method (Sanger *et al.*, 1977). We analyzed both strands in most of the cases by use of double-stranded form and an appropriate primer. The triplets which were changed by mutations are shown in Table II. During the development of the new technique for mutagenesis, we obtained 19 mutants exhibiting altered cross-

reaction to either ARM191 or ARM193 from among the 52 plaques tested (Table I). So far as defined, all mutations were the substitution of amino acid(s) caused by base substitution(s) (Table II). Some of the mutants have a second (third) base substitution which does not affect the amino acid sequence. Of the base substitutions defined, 83% are transitions and 17% transversions.

On the first screening, the addition of dITP appeared to have only a little effect on the PCR mutagenesis (Table I). However, when we characterized the mutants isolated, we found that the addition of dITP is essential for this mutagenesis (Table I). Without dITP or with 0.2 μM dITP, we picked up 5 and 21 plaques, respectively, on the first screening, but obtained only 3 kinds of mutants. When dITP was added at 200 μM, we picked up 21 plaques from among about 2000 plaques expressing the fused recA polypeptide, and from these groups we finally isolated 11 mutants which were categorized into 10 kinds. Three of them (mutants 30, 33, and 36) had base substitutions at two or three sites (see Table II). These findings indicate the significant enhancement of the yield and variation on mutagenesis with dITP, and the very high yield of base substitutions under an appropriate set of conditions.

Some mutants have base substitutions at the same sites. Except mutants 23 and 34 (substitution of Ser[333]), such overlapped mutants were isolated from the same PCR preparations. Thus, these overlapped mutations seem to be created at an early cycle in PCR and amplified during the procedure.
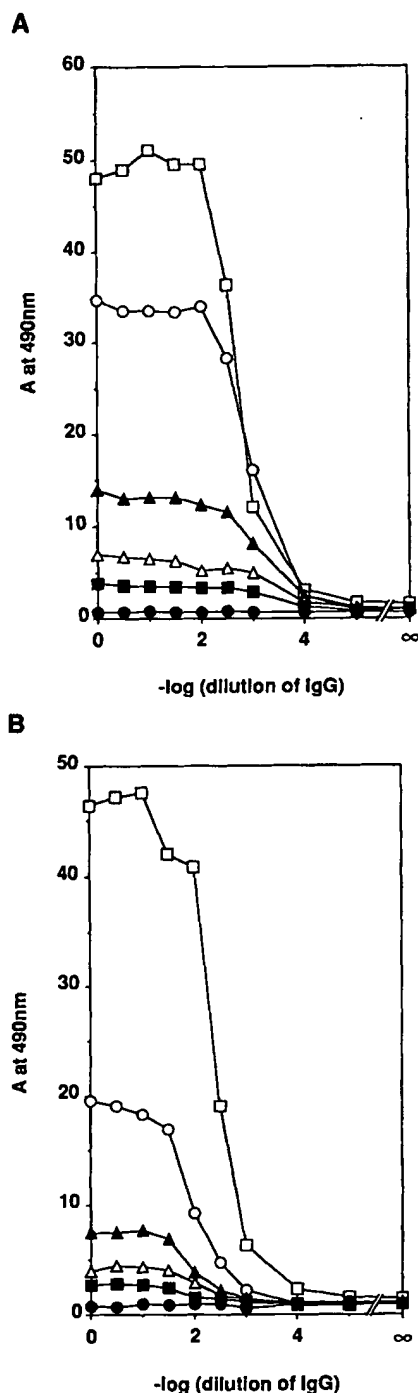
A

-log (dilution of IgG)

B

-log (dilution of IgG)

FIG. 5. **Variation of the antigen concentration on ELISA does not change the amount of IgG giving a half-maximum signal.** The wells of a microtiter plate were coated with the purified recA protein at the indicated concentrations. On the other hand, the concentration of the anti-recA protein IgG (ARM191 in *A*; ARM193 in *B*) was adjusted to 30 μg/ml ("nondilution"), and then a series of dilutions of the IgG was put into individual wells. The bound IgG was measured by ELISA. The concentrations of the recA protein were as follows: □, 2 μg/ml; ○, 0.2 μg/ml; ▲, 0.1 μg/ml; △, 0.05 μg/ml; ■, 0.02 μg/ml; ●, without recA protein.

Except for in these cases, base substitutions appeared to be introduced at random in the amplified DNA.

Cross-reaction of Mutant recA Proteins—We constructed a plasmid (pMI996; Fig. 2) in which the wild type *recA* gene was under the control of the *Tac* promoter on a multicopy plasmid (a derivative of pKK223-3 (Brosius and Holy, 1984). We replaced the *Eco*RI-*Eco*RI region encoding the C-terminal

A　　　　　　　　**B**

-log (dilution of IgG)　　　-log (dilution of IgG)

C　　　　　　　　**D**

-log (dilution of IgG)　　　-log (dilution of IgG)
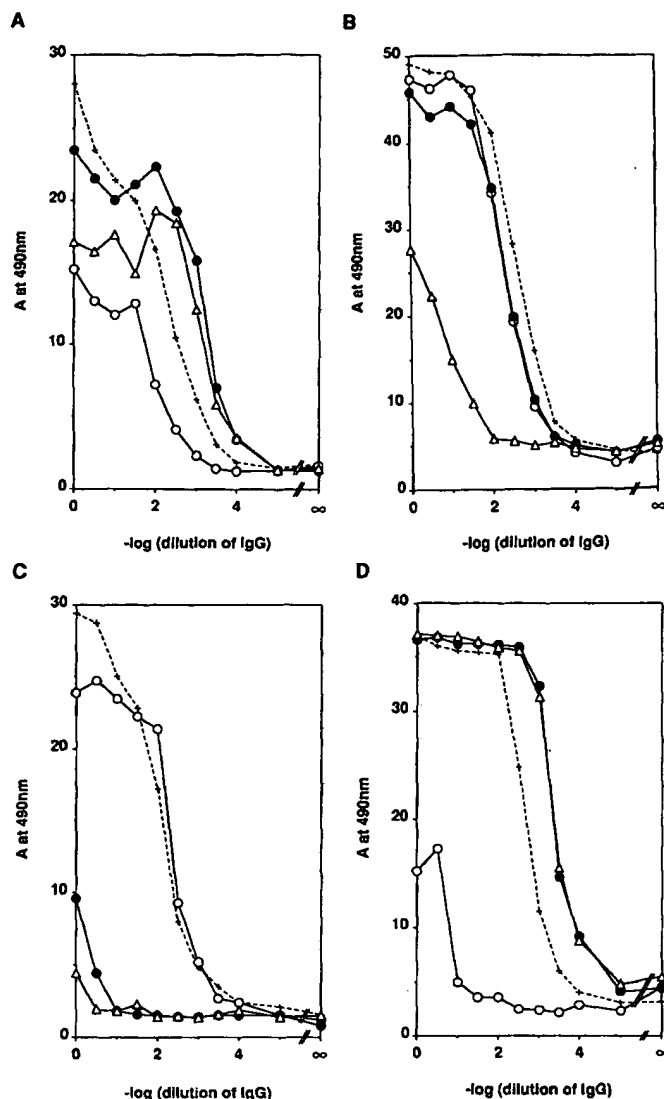
FIG. 6. **Examples of ELISA for testing the cross-reaction of mutant recA proteins to anti-recA protein IgGs, ARM191, ARM193, and MAb156.** Cell-free extracts were prepared from cells in which the mutant *recA* genes were expressed; *A*, wild type; *B*, mutant 38; *C*, mutant 32; *D*, mutant 31. The cell-free extracts were put into individual wells of a microtiter plate to allow the adsorption of proteins. Then, the cross-reaction to each of the IgGs was examined by ELISA, as described in the legend to Fig. 5. The IgGs used were: ●, ARM191; ○, ARM193; △, MAb156. Anti-recA protein IgG, ARM414, of which epitopes is located between Glu^{233} and Lys^{256} (Ikeda *et al.*, 1990) was used as a positive control (+).

93 amino acids of the wild type recA polypeptide with the *Eco*RI fragments on which we found mutation(s) (Fig. 2). Then, a mutant of *E. coli* in which the whole *recA* gene was deleted was transformed with these plasmids. The expression of the mutant *recA* genes was induced and cell-free extracts were prepared, followed by quantitative assaying (ELISA) for cross-reaction with ARM191 and ARM193. For comparison of the extent of cross-reaction of a tested IgG with the recA protein, we determined the amount of the IgG giving a half-maximum signal on ELISA. As shown in Fig. 5, variations in the amount of recA protein did not significantly change the amount of the IgG which gave the half-maximum signal on ELISA. We calculated the amounts of ARM191 and ARM193 giving a half-maximum signal to be 0.0277 ($\sigma = 0.017$) and 0.29 ($\sigma = 0.10$) μg/ml, respectively, from the data in this figure.
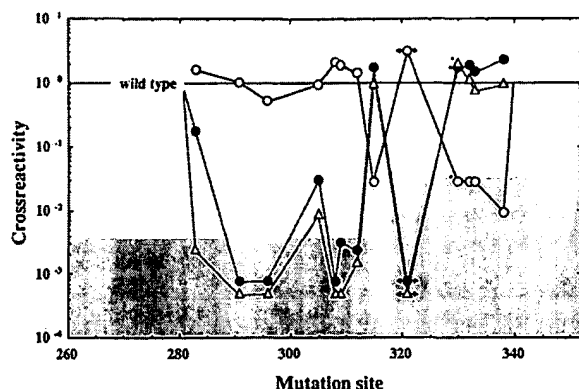
FIG. 7. **Mutation sites and alterations in cross-reactivity.** The *numbers* under the *abscissa* are the amino acid positions from the N terminus of the recA polypeptide. Cross-reactivity is defined as follows: (the amount of an IgG giving the half-maximum value on ELISA with wild-type recA protein)/(the amount of an IgG giving the half-maximum value on ELISA with mutant recA protein). Thus, it shows the deficiency in the cross-reaction. ●, cross-reaction with ARM191; ○, that with ARM193; △, that with MAb156. *Symbols within the shadowed area* indicate that no cross-reaction was detected (within the limit of the measurement, which is indicated by each *symbol*). The *symbols* with a *horizontal arrow* around position 320 are those for a mutant having two amino acid substitutions at positions 319 and 323, and those with * at position 330 are those for a mutant having two amino acid substitutions at positions 294 and 330.

Examples of ELISA with mutant recA proteins as well as the wild type protein are shown in Fig. 6. The values calculated on ELISA are listed in Table II. In order to obtain the half-maximum signals on ELISA, all proteins (except mutant 38) which had been determined to be mutants by immunoblotting experiments were shown to require at least 10-fold more IgG ARM191 or ARM193 than the average for plus proteins (Fig. 7). The results shown in Fig. 7 clearly indicate that the mutants affecting the cross-reaction with ARM191 and those affecting that with ARM193 are separately clustered, and only slightly overlap each other (between positions 315 and about 320). This conclusion is consistent with the absence of competition in the binding of these IgGs to the recA protein (Fig. 3).

Comparison of MAb156 with ARM191 and ARM193— MAb156 was isolated by Karu and Allen (Karu and Allen, 1982). Since it was assumed that the epitope of this antibody is located near the C terminus of the recA polypeptide,[2] we examined the cross-reaction of MAb156 with the mutant recA proteins isolated in the present study. All of the mutations affecting the cross-reaction with ARM191 also affected the cross-reaction with MAb156, but none of the mutations affecting the cross-reaction with ARM193 did. This suggests that the epitope for MAb156 and that of ARM191 are similar, but the following results show that they are not identical. Most of the mutations abolishing the cross-reaction with ARM191 also abolished that with MAb156 (Fig. 7), but substitution of Leu^{283} by Pro in mutant 38 strongly interfered with the cross-reaction with MAb156, but not so much with that with ARM191 (Figs. 6B and 7). The difference in the mode of cross-reaction between ARM191 and MAb156 was also observed with a mutant recA protein having a substitution of Ala^{305} by Gly (Fig. 7).

DISCUSSION

Region-specific PCR mutagenesis is an efficient tool for introducing random base substitution mutations specifically

---

[2] A. J. Clark and A. Karu, personal communication.

in a region defined by a pair of primers. From among the plaques expressing the fused *recA* gene, we obtained mutants of the *recA* gene at the frequency of 0.5% under the optimized conditions. Using this mutagenesis, we identified regions of the recA polypeptide in which amino acid substitutions prevent the cross-reaction with ARM191, ARM193, and/or MAb156. The region for ARM191 and that for ARM193 are different, but slightly overlap each other; *i.e.* that for the former IgG comprises positions 283 through about 320, and that for the latter positions through 315–338. Since ARM191 and ARM193 showed no competition, Thr^{315}, and Ile^{319} and Ile^{319} and/or Val^{323} would be located on different sides of a local structure or the whole molecule of the recA polypeptide.

The substitution of an amino acid might interfere with the cross-reaction with an IgG directly or through a very local change in the tertiary structure, or indirectly through extensive alteration of the tertiary structure of the recA protein. The Leu^{283} to Pro substitution in mutant 38 could cause gross alteration of the tertiary structure of the recA protein. The clustering of other amino acid substitutions in a particular region is favorable for the first two possibilities rather than the last one. ARM193 was shown to prevent the self-assembly of the recA protein and ARM191 to inhibit the binding of double-stranded DNA. Thus, the active sites involved in these functions would be located in the space at or around the relevant epitope. We are testing these possibilities by examining the effects of the mutant recA proteins isolated in this study and those constructed by another round of region-specified PCR mutagenesis. Determination of the epitope loci and the inhibitory effects of these IgGs, together with the tertiary structure of the recA protein, will facilitate the understanding of the function of the protein in relation to its structure.

The technique of region-specified random base substitutions involving the use of PCR employed in this study is very useful not only for epitope mapping, as described in this paper, but is also widely useful for studies on the function of a gene and an enzyme or protein, because of the flexibility as to specifying a target region, and the high yield of the random base substitutions.

REFERENCES

Berger, S. L., and Kimmel, A. R. (1987) *Guide to Molecular Cloning Techniques. Methods in Enzymology,* Vol. 152, Academic, Orlando
Brosius, J., and Holy, A. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 6929–6933
Eckert, K., and Kunkel, T. A. (1990) *Nucleic Acid Res.* **18,** 3739–3744
Ikawa, S., Kamiya, N., and Shibata, T. (1989) *J. Biol. Chem.* **264,** 21167–21176
Ikeda, M., Makino, O., and Shibata, T. (1990) *J. Biol. Chem.* **265,** 8948–8956
Karu, A. E., and Allen, L. (1982) *Biochimie (Paris)* **64,** 723–729
Makino, O., Shibata, Y., Maeda, H., Shibata, T., and Ando, T. (1985) *J. Biol. Chem.* **260,** 15402–15405
Makino, O., Ikawa, S., Shibata, Y., Maeda, H., Ando, T., and Shibata, T. (1987) *J. Biol. Chem.* **262,** 12237–12246
Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Martin, F. H., and Castro, M. M. (1985) *Nucleic Acid Res.* **13,** 8927–8938
Morishima, N., Nakagawa, K., Yamamoto, E., and Shibata, T. (1990) *J. Biol. Chem.* **265,** 15189–15197
Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. (1985) *Science* **230,** 1350–1354
Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
Shibata, T., Cunningham, R. P., and Radding, C. M. (1981) *J. Biol. Chem.* **256,** 7557–7564
Shibata, T., Ikeda, M., Makino, O., and Ikawa, S. (1991) *Biochimie (Paris)* **73,** 209–218
Uhlin, B. E, and Clark, A. J. (1981) *J. Bacteriol.* **148,** 386–390
Vieira, J., and Messing, J. (1987) *Methods Enzymol.* **153,** 3–11
Young, R. A., and Davis, R. W. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 1194–1198